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Acceleration of diabetic wound healing with chitosan-crosslinked collagen sponge containing recombinant human acidic fibroblast growth factor in healing-impaired STZ diabetic rats

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Abstract

In order to develop a better wound-dressing to enhance diabetic wound healing, we have examined the biochemical and biophysical features of chitosan-crosslinked collagen sponge (CCCS) and pre-clinically evaluated the CCCS containing recombinant human acidic fibroblast growth factor (CCCS/FGF) in accelerating diabetic wound healing as compared to collagen sponge alone and FGF alone. Collagen crosslinked with chitosan showed several advantages required for wound dressing, including the uniform and porous ultrastructure, less water-imbibition, small interval porosity, high resistance to collagenase digestion and slow release of FGF from CCCS/FGF. Therapeutic effect of the new wound-dressing containing FGF (i.e.: CCCS/FGF) on diabetic wound healing was examined in type 1 diabetic rat model in which hyperglycemia was induced by single dose of streptozotocin (STZ) and persisted for two months. The CCCS/FGF provided the most efficiently therapeutic effect among various treatments, showing the shortest healing time (14 days in the CCCS/FGF-treated group as compared to 18～21 days in other treatment groups), the quickest tissue collagen generation, the earliest and highest TGF-β1 expression and dermal cell proliferation (PCNA expression). All these results suggest that CCCS/FGF is an ideal wound-dressing to improve the recovery of healing-impaired wound such as diabetic skin wound, which provides a great potential use in clinics for diabetic patients in the future.

Keywords: Recombinant human acid FGF; Diabetic wound healing; Chitosan; Collagen sponge; Wound dressing

Introduction

The prevalence of diabetes has increased tremendously world-wide, and diabetic complications have become a serious issue for public health. One of these complications is the impaired-wound healing of diabetic patients (Brem and Tomic-Canic, 2007; Pavlovic et al., 2007). Lack of cellular and molecular signals required for normal wound-repair process such as angiogenesis, granulation tissue formation, epithelialization, and remodeling may be a major contributing factor to the poor healing of diabetic wound (Blakytny et al., 2000; Jude et al., 2002; Blakytny and Jude, 2006; Brem and Tomic-Canic, 2007). Cytokines, especially various growth factors, provide the cellular and molecular signals necessary for normal healing process, but are deficient in diabetic wounds (Blakytny et al., 2000; Jude et al., 2002;

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Blakytny and Jude, 2006; Brem and Tomic-Canic, 2007). Topical application of several growth factors to stimulate fibroblast and endothelial cell proliferation to heal the impaired wound might increase the rate and degree of granulation tissue and capillary formation, and thus stimulate wound healing (Inoue et al., 1998; Lee, 2005; Cheng et al., 2007).

Both acidic and basic fibroblast growth factor (aFGF and bFGF or called FGF-1 and FGF-2) have many biological activities that stimulate the proliferation of fibroblasts and capillary endothelial cells, thus promoting angiogenesis and wound healing (Gerwins et al., 2000; Li et al., 2006, 2007; Huang et al., 2007). However, the application of both aFGF and bFGF in the therapy of the healing-impaired wound was not always successful (Tsuboi and Rifkin, 1990; Papanas and Maltezos, 2007), due to its high diffusibility and short half-life in vivo. Therefore, new approaches to stably deliver growth factors locally into the wound tissues are required for applying FGFs in clinics to improve the wound healing.

Two early studies (Marks et al., 1991; Ono et al., 1999) found that collagen sponge was useful not only as a carrier of cytokines, including FGF, but also as for the quick closure of chronic wounds, thereby preventing contracture, one of the most challenging problems in treating skin wound. Application of collagen sponge (Marks et al., 1991; Ono et al., 1999; Nakanishi et al., 2005; Nagato et al., 2006) and photocrosslinking chitosan hydrogel containing FGF to chronic wounds has been explored separately (Obara et al., 2003, 2005a). The latter was found to be a viscous solution and easily crosslinked, resulting in an insoluble hydrogel within 30 seconds. When FGF was added into chitosan hydrogel, major proportion of FGF molecules retained in the chitosan hydrogel remained biologically active and releasable upon in vivo application, resulting in a significantly enhanced and prolonged vascularization effect (Obara et al., 2003, 2005a). However, since each of collagen or chitosan alone has certain unsatisfied features (Marks et al., 1991; Ono et al., 1999; Nakanishi et al., 2005; Nagato et al., 2006), mixture of collagen with chitosan has been explored to optimize the matrix for efficiently delivering to and stably localizing in the target tissues of these cytokines, and also experimentally used as a new wound-dressing material for damaged tissue recovery in various organs (Taravel and Domard, 1993, 1995, 1996; Sionkowska et al., 2004). However, the new dressing has not been tested for its improvement of diabetic wound healing.

The purpose of the present study was to assess the biochemical and biophysical improvement of the chitosan-crosslinked collagen sponge (CCCS) containing recombinant human aFGF (rhaFGF), i.e., CCCS/FGF, as the new wound dressing for the therapeutic effect on the healing-impaired skin-wound in streptozotocin (STZ)-induced diabetic rats. The diabetic wound healing was found to be significantly improved by CCCS/FGF, as compared to other approaches, suggesting the potential of CCCS/FGF as a new wound dressing for clinically application to diabetic wound healing.

Material and methods

Reagents

Pepsinum, MTT assay reagents, collagenase, and STZ were purchased from Sigma Inc. (USA), and pancreatin and RPMI-1640 were from Gibco Inc. (USA). Blood sugar test strips, and antibodies against TGF-β1 and PCNA were purchased from LifeScan Inc. (USA) and Santa Cruz (USA), respectively. All other reagents including calf serum (Sijiqing of Hangzhou, China), hydroxyproline kit (JianCheng of Nanjing, China), chitosan (Guangzhou Chemical Agent, Co., China), urine sugar test strips, and β-actin, SABC kit, ELISA kit, DAB developer, goat anti-rabbit IgG-HRP (Boshi De Inc., Wuhan, China) were purchased from different companies in China. Recombinant human acidic FGF (rhaFGF) was obtained from the Center of Medicine and Biotechnology R& D at the Jinan University (concentration: 1 mg/ml; activity: 10^5 U/ml; lot number: 20040701, 20061126). Balb/c mouse fibroblast (3T3) cell line and normal rat kidney epithelial (NRK52E) cell line were obtained from Jinan University, and Sprague–Dawley (SD) rats from Experimental Animal Center of Guangdong Province.

Extraction of collagen, and preparation of collagen sponge with and without chitosan-crosslinking

After the grease was removed, the fresh bovine tendo calcaneus was washed clearly with distilled water and then put into −20 °C refrigerator. After they sterilized with 75% alcohol for 30 minutes and washed with sterile distilled water, the bovine tendo calcaneus was bleached with 0.5 M glacial acetic acid for more than 24 hours at 4 °C, with periodically stirring. Then they were comminuted, homogenized, and suspended in 0.5 M acetic acid containing 1% (w/w) pepsinum for more than 24 hours at 4 °C allowing tendo calcaneus being completely dissolved. After centrifugation, the supernatant was dialyzed and the precipitate was dissolved in 0.5 M glacial acetic acid again. The samples were filtered to obtain the pure collagen solutions for lyophilization.

Collagen solutions in three different concentrations of 0.11 mg/ml, 0.22 mg/ml, and 0.41 mg/ml were crosslinked with 1% chitosan at ratios of 10:1, 5:1, 1:1, respectively. Therefore, 9 sets of chitosan-crosslinked collagen sponge (CCCS) and 3 sets of collagen sponge alone were made.

To make collagen sponge and CCCS with FGF (i.e.: collagen/FGF and CCCS/FGF), rhaFGF was added into the collagen solution at the concentrations of 1, 2, or 4 μg FGF/2.5 cm^3 collagen sponge (5 cm × 5 cm × 0.1 cm for length, width and thickness), and fully mixed up and desiccated to form the collagen/FGF or CCCS/FGF. Both collagen/FGF and CCCS/FGF were sterilized with exposure to C_O^{60} overnight at a dose of 8000 Gy.

Characteristics of CCCS with and without rhaFGF

Test for the water-imbibition rate

CCCS was weighted as W1 and put into a container filled with saline and degasified for more than 10 minutes to make

sure that saline had engorged the port of sponges. Then CCCS was taken out and weighted as W2. The water-imbibition rate of CCCS was calculated according to the following equation: water-imbibition rate (%) = {W2(g)-W1(g)}/W1(g)*100.

Test of interval porosity

A 5 ml glass bottle was fully filled with alcohol and then weighted W1. The CCCS that has been weighted as WS was immersed into the alcohol and degasified to make sure that alcohol had engorged the port of the sponges. The bottle was fully filled up with alcohol again to avoid the spilt out of the liquid when CCCS was put in, and weighted as W2. The CCCS engorged with alcohol was taken out and the glass bottle containing the rest of alcohol was weighted as W3. The interval porosity of CCCS was calculated according to the following equation: interval porosity = (W2-W3-WS)/(W1-W3)*100.

Degradation of CCCS

Thirty-six beakers were filled with 100 ml of PBS (after steam sterilization) with 2 mg collagenase. The solutions were mixed up at room temperature and put in 4 °C refrigerator overnight. CCCSs were put into the above-mentioned beakers after weighing. Triplicate 1-ml solutions were sampled into eppendorf tubules at 0, 6, and 12 hours, and day 1 until day 60, respectively, to measure degraded collagen from CCCS using hydroxyproline kit based on the manufacture's protocol.

Releasing rate of rhaFGF from CCCS

Thirty-six beakers were filled with 50 ml of PBS (after steam sterilization) and with CCCS/FGF for each beaker. Triplicate 1-ml solutions were sampled into eppendorf tubules at 0, 2, 6, and 12 hours, and also day 1 until day 14, respectively, to measure the concentrations of FGF released from CCCS/FGF, using ELISA kit based on the manufacture's protocol.

Experiments for cell growth and proliferation

Observation of cell growth status in the collagen sponge with scanning electron microscope

After cryodesiccation, sterilized collagen sponges were placed into 6-well plates. Cell suspensions at 10^4-10^6 /ml for 3T3 cells and NRK52E cells were planted into each well of the plates to be incubated at a 5% CO_2 humidified atmosphere and 37 °C for 72 hours. After incubation, the state of cell growth in the sponge was examined under scanning electron microscopy (SEM).

Evaluation of the cell proliferation effect by methylthiazol tetrazolium

Collagen sponges with and without rhaFGF (80 ng/ml) were prepared in 96-well plates as described above. Five thousand 3T3 cells or NRK52E cells in a volume of 100 μl were planted into each well, respectively, and incubated at 5% CO_2 humidified atmosphere and 37 °C for 72 hours. Then methylthiazol tetrazolium (MTT) assay was performed to examine the cell viability based on the kit instruction by monitoring the visible absorbance of each sample at 570 nm.

Animal model and treatments

Type 1 diabetes model

Male SD rats, weighing 180 to 220 g were given a single i.p injection of 62 mg/kg STZ dissolved in sodium citrate buffer (pH. 4.5), and 3 days later, whole-blood glucose obtained from rat tail-vein was monitored using SureStep Complete Blood Glucose monitor (LifeScan Inc. Milpitas, CA). STZ-treated rats with whole-blood glucose levels higher than 16.7 mM were considered diabetic. Rats serving as vehicle controls were given the same volume of sodium citrate. All animal procedures were approved by the University Animal Case and Use Committee, which is certified by the Chinese Association of Accreditation of Laboratory Animal Care.

Establishment of the cutis trauma model

The cutis trauma model was produced in these diabetic rats at 2 months after the onset of diabetes. Rats were etherized for anesthesia and denuded with 8% Na_2S. A round iron with the diameter of 1.8 cm was water-boiled beforehand and placed on each side of the rat back for 30 seconds respectively, to make deep II scald. The whole skin layer of scald was cut off 3 days later to make the cutis trauma model (simply called skin wound in the following parts).

Randomization and administration

There were two sets of animal experiments: For the first set of animal experiment to test the therapeutic effect of collagen sponge with FGF, 70 rats (except 3 rats which were died and 2 rats which failed to become skin wound) were divided into 7 groups: Control (skin-wound non-diabetic rats treated with saline, n = 6), Diabetes (skin-wound diabetic rats treated with saline, n = 9), D/collagen (skin-wound diabetic rats treated with collagen and saline, n = 10), D/FGF (skin-wound diabetic rats treated with 4 μg FGF in 2.5 ml saline to cover wound surface, n = 10), D/collagen/FGF(L) (skin-wound diabetic rats treated with collagen sponge containing 1 μg FGF/2.5 cm^3, n = 10), D/collagen/FGF(M) (skin-wound diabetic rats treated with collagen sponge containing 2 μg FGF/2.5 cm^3, n = 10), D/collagen/FGF(H) (skin wound diabetic rats treated with collagen sponge containing 4 μg FGF/2.5 cm^3, n = 10). One single administration of various treatments was given once a week.

For the second set of animal experiment to test the therapeutic effect of CCCS/FGF, 10 non-diabetic and 60 diabetic rats (two months after hyperglycemia) were made for skin wound and randomly divided into 7 groups except for control group: Control (non-diabetic rats treated with saline, n = 10), Diabetes (diabetic rats treated with saline, n = 10), D/CCCS (diabetic rats treated with CCCS and saline, n = 10), D/FGF(L) (diabetic rats treated with 1 μg FGF in 2.5 ml saline to cover wound surface, n = 10), D/FGF(H) (diabetic rats treated with 4 μg FGF 2.5 ml saline, n = 10), D/CCCS/FGF(L) (diabetic rats treated with CCCS with 1 μg FGF/2.5 cm^3, n = 10), and D/CCCS/FGF(H) (diabetic rats with skin wound and treatment of CCCS with 4 μg FGF/2.5 cm^3, n = 10). Since treatment with rhaFGF at 1 ~ 4 μg for the similar size skin-wound has been used once a week extensively in Chinese hospitals as one of the effective therapy for diabetic wound healing, we used D/FGF

(L) and D/FGF(H) as the routine therapy controls in the current study. For all groups, one single treatment was given once a week, same as the first set of experiment.

Evaluation of the wound healing

The inflammatory reaction in wound surface, its ambience and the wound closure status were evaluated every day after various treatments. Healing size was evaluated by photographing the wound area at a close and fixed distance. Remaining unhealed-wound size was measured based on the image using UTHSCSA Image Analyzing Tool. Healing time of wound was counted when unhealed wound area was less than 30 mm^2.

Histopathological and immunohistochemical examination

Rats were sacrificed on day 7, 14 and 28 after various treatments. Moiety of the trauma samples were put into formaldehyde (10%) and embedded in paraffin. Skin tissues were cut into 4 μm sections for histopathological examination by hematoxylin-eosin (HE) staining, for collagen formation assay by Masson's trichrome staining, for tissue fibrosis and recovery by immunohistochemical staining of tissue growth factor-β1 (TGF-β1), and proliferating cell nuclear antigen (PCNA). Paraffin-embedded sections were deparaffinized and redehydrated, and then were washed with water and stained by HE. HE-stained sections were observed with a Nikon Eclipse E400 light microscope.

For Masson's trichrome staining, tissue section were treated as describe above for the deparaffinization and redehydration and then stained based on the instruction of Masson's Trichrome Staining kit. By this staining, the collagen and nuclei would be stained as blue and black, while muscle and keratin would be stained as red.

For immunohistochemical staining, the deparaffinized and redehydrated tissue sections were inactivated the endogenous peroxidase by an incubation with 3% H_2O_2 for 10 minutes. To recover antigen, these sections were put into 10 mM citrate buffer solution (pH 6.0) and heated in the microwave oven twice. The slides were then washed with PBS (pH7.2-7.6) twice. Non-specific binding sites were blocked with 5% BSA in TBS for 20 minutes. After the redundant liquid was discarded, the sections were incubated with primary antibodies at 4 °C overnight or at room temperature for 1 hour and washed with PBS. Then the slides were incubated with biotinylated secondary antibody for 20 minutes, followed by incubation with streptavidin-HRP for 20 minutes. The antibody binding sites were visualized by incubation with a DAB-H_2O_2 solution. The slides were counterstained for 1 minute with hematoxylin and then dehydrated with sequential ethanol for sealing and microscope observation.

Western blot analysis

Tissues were homogenized in lysis buffer, and total tissue proteins were collected by centrifuging at 12,000 rpm at 4 °C in a Beckman GS-6R centrifuge for 10 minutes. The protein concentration was determined and the sample was then mixed with loading buffer (40 mM Tris-HCl pH 6.8, 1% SDS, 50 mM DTT, 7.5% glycerol, 0.003% bromophenol blue) and then subjected to electrophoresis on a 16% SDS-PAGE gel at 120 V, followed by transfer to PVDF membrane. After blocked with 5% nonfat milk for 2 hours at room temperature, the membrane was washed and incubated with primary antibody (anti-rabbit, at a dilution of 1:1000) for 1 hour at room temperature and kept in 4 °C overnight. Membranes were then washed three times with TBS-T containing 0.05% tween20 and reacted with secondary HRP-conjugated antibody for 1 hour. Antigen-antibody complexes were visualized by DAB. Expression of β-actin was used as a control.

Statistical analysis

All experimental values are presented as means ± SD. The data were analyzed using ANOVA, and then paired *t*-test was used to analyze the difference between the groups. P value at less than 0.05 was considered statistically significant.

Results

Evaluation of collagen sponge

SEM examination displayed that collagen sponge was porous uniformly (Fig. 1A), and bio-safety test showed that both 3T3 cells (Fig. 1B) and NRK52E cells (Data not shown) were growing well. Inclusion of rhaFGF in the collagen sponge stimulated cell proliferation for both cell lines, measured by MTT assay (Fig. 1B, C). In Fig. 1B, C, it is also shown that collagen sponge containing FGF (group VII) provides the most efficient effect in stimulating cell proliferation among various groups, although inclusion of both collagen sponge and FGF separately (either FGF first and then collagen or collagen first and then FGF were separately added into wells) also have some stimulating effects on the cell proliferation (groups V and VI).

To evaluate the potential use of the rhaFGF-containing collagen sponge in clinics, hyperglycemic animal model was induced by single dose of STZ at 62 mg/kg in SD rats. These diabetic rats were hyperglycemic until experimental termination (Fig. 2A) and showed a slow body-weight gain during this period (Fig. 2B), consistent with our previous studies in diabetic rat and mouse models (Cai et al., 2000, 2002, 2005, 2006). At 2 months after hyperglycemia, skin burn was made and mechanical removal of the burn skin to make a deep trauma (skin wound) (Fig. 2C). Collagen sponge containing different concentrations of rhaFGF at 1, 2, 4 μg/2.5 cm^3 was used as wound dressing to treat the healing-impaired skin wound in these STZ-diabetic rats. Healing time of the diabetic wound was slightly shortened in the rats with collagen/FGFs [D/collagen/FGF(L), D/collagen/FGF(M), D/collagen/FGF(H)] as compared to groups of Diabetes, D/collagen, and D/FGF (Fig. 2D). The remaining

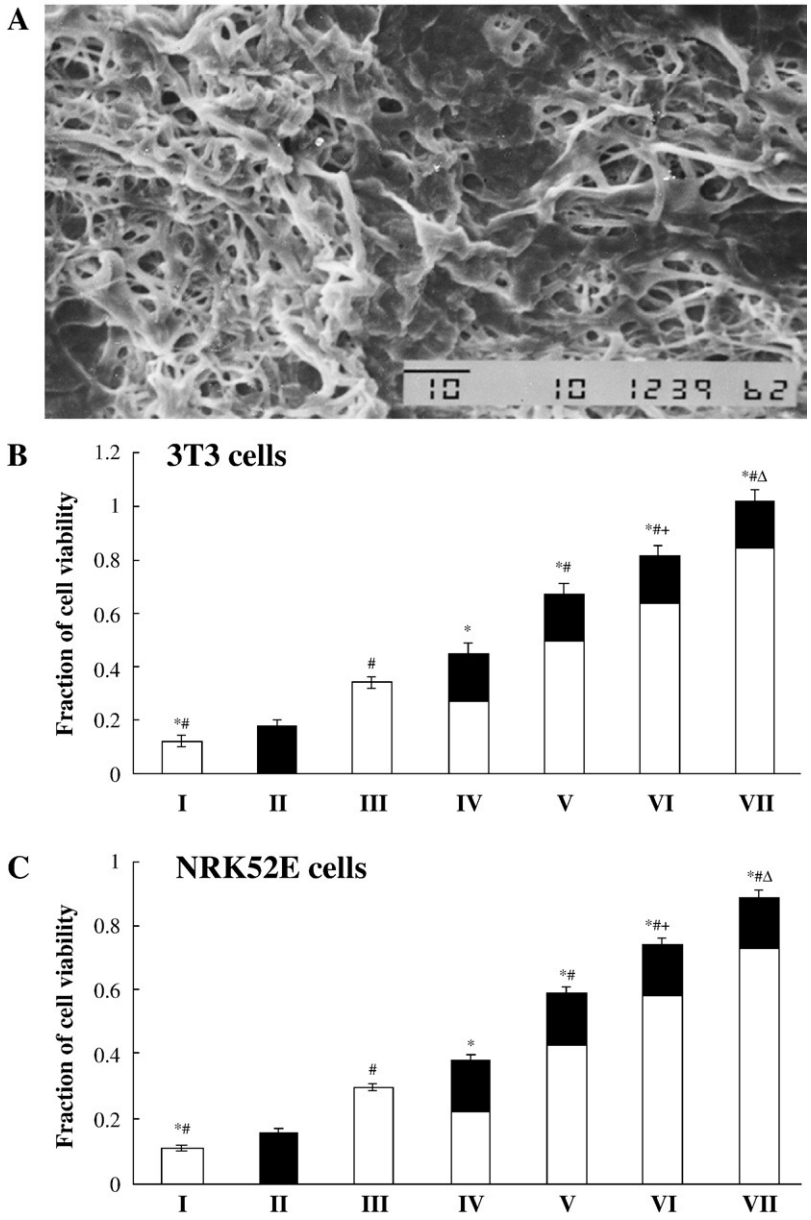

Fig. 1. Cell growth evaluation for collagen sponge with and without rhaFGF using 3T3 and NRK52E cells. Sterilized collagen sponge added with 3T3 cells were incubated under the conditions described in *Materials and Methods* for 72 hours, and then cell growth status was examined under SEM (A). In panels of B and C, either 3T3 cells or NRK52E cells were added at 1×10^3 cells in 100 μl into 96-well plates in which 7 groups from I to VII were included: I (wells included medium only), II (wells included collagen sponge only), III (wells included medium with 80 ng/ml rhaFGF), IV (wells included medium with collagen sponge), V (wells included medium with sponge first and then 80 ng/ml FGF, added separately in order), VI (wells included medium with 80 ng/ml FGF first and then sponge, added separately in order), and VII (wells included medium with collagen sponge containing 80 ng/ml FGF). The cells in the 96-well plates were incubated for 72 as described in *Materials and Methods*, and then measuring visible light absorption at 570 nm with spectrometer performed MTT assay. White parts of the column indicate the absorption attributed by cells and black parts of the column indicate the absorption attributed by collagen sponge. *, $p<0.05$ vs group III; #, $p<0.05$ vs group II; +, $p<0.05$ vs group V; Δ, $p<0.05$ vs group VI.

unhealed wound sizes in different groups are summarized in Table 1, which indicates that in non-diabetic rats (Control), skin wound was completely healed at 14 days after saline treatment, while it was not healed in diabetes treated with saline until 28 days (Diabetes). In contrast, various treatments for diabetic wound all significantly reduced the unhealed wound size (Table 1), with the fastest healing in the groups of diabetic rats treated with collagen sponge containing various concentrations of rhaFGF (completely healing time from 18 to 21 days).

However, it should be noted that there was no FGF-dose dependence for the healing time and unhealed wound sizes among groups with different concentrations of rhaFGF contained in collagen sponge (Fig. 2D, Table 1). We assumed that rhaFGF contained in collagen sponge might be quickly released into wound area in a very short time period so that even

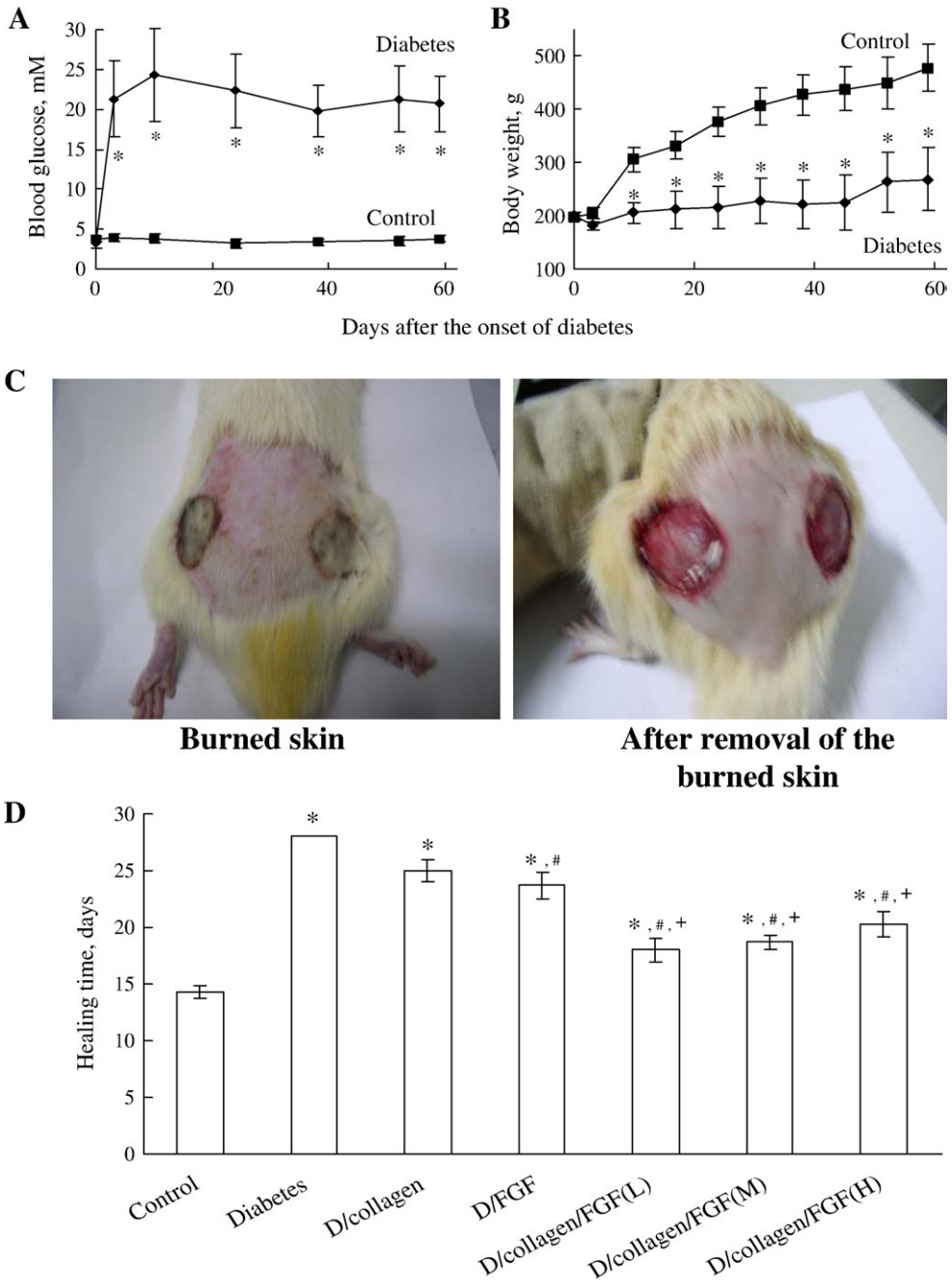

Fig. 2. STZ-induced type 1 diabetic model and therapeutic effects diabetic wound healing under different conditions. SD rats were treated with single dose of STZ to induce hyperglycemia that was lasted until the time of the experiment (A). Diabetic rats showed the less body-weight gain as compared to non-diabetic rats (B). Panel C indicates the status of boiled iron-induced burn skin before and after removal of the burned skin. Panel D indicates the healing time of the diabetic wound under different treatment conditions. Group conditions have been given in the text part. *, $p<0.05$ vs Control; #, $p<0.05$ vs Diabetes; +, $p<0.05$ vs D/FGF.

high concentration of FGF contained in sponges could not provide a longer therapeutic effect. Correspondingly, to modify the sponge for slowly releasing rhaFGF from sponge remains required.

General evaluation of the chitosan-crosslinked collagen sponge (CCCS)

To reach the goal mentioned above, collagen in three different concentrations (0.11 mg/ml, 0.22 mg/ml, and 0.41 mg/ml) was crosslinked with 1% chitosan solution (i.e.: chitosan-crosslinked collagen sponge, CCCS) at three ratios of 10:1, 5:1 and 1:1 (collagen/chitosan). SEM observation revealed that the CCCS are uniformly porous, the size of which is negatively correlated with the increases in both collagen and chitosan concentrations, i.e.: the smallest porosity in the CCCS composed of 0.41 mg/ml collagen at 1:1 ratio of collagen to chitosan (Fig. 3A).

Analysis of CCCS imbibition by the method described in *Materials and Methods* indicates that both concentrations of

Table 1
Unhealed wound size (mm^2) at different times after starting treatments

Day/group	Control (n=6)	Diabetes (n=9)	D/collagen (n=10)	D/FGF (n=10)	D/collagen/FGF(L) (n=10)	D/collagen/FGF(M) (n=10)	D/collagen/FGF(H) (n=10)
0	254	254	254	254	254	254	254
3 d	205.3±9.2*	233.6±13.4	220.6±13.3*	203.5±9.1*	158.3±4.7*#+	167.2±5.8*#+	183.2±6.1*#+
7 d	108.8±4.8*	206.8±9.8	165.3±4.7*	134.3±4.3*	99.2±3.1*#+	103.4±4.5*#+	115.6±4.9*#+
10 d	77.6±2.2*	188.3±6.6	132.7±4.1*	112.2±2.9*	75.3±1.9*#+	78.5±2.6*#+	93.4±2.1*#+$
14 d	13.4±1.6*	169.6±4.3	99.3±2.7*	79.7±1.8*	37.5±1.1*#+$	43.1±1.2*#+	59.3±1.6*#+$
18 d	0	143.7±3.9	68.4±1.9*	56.3±1.5*	11.2±0.7*#+$	13.3±0.6*#+	21.5±0.7*#+$
21 d		112.2±3.1	37.9±1.5*	15.8±0.8*	3.2±0.3*#+$	4.2±0.4*#+	7.9±0.3*#+$
25 d		76.9±1.9	11.5±0.9*	0	0	0	0
28 d		34.3±1.7	0				

Notes: *, $p<0.05$ vs Diabetes; #, $p<0.05$ vs D/collagen; +, $p<0.05$ vs D/FGF; $, $p<0.05$ vs D/collagen/FGF(M).

collagen and chitosan negatively affect the imbibition of CCCS: the higher the concentration of either collagen or chitosan in CCCS, the less the imbibition of CCCS (Fig. 3B). Determination of the interval porosity of CCCS by the method described in *Materials and Methods* showed that concentrations of collagen and chitosan both are also negatively correlated to the interval porosity of CCCS, i.e.: the higher the concentration of either collagen or chitosan in CCCS, the smaller the interval porosity of CCCS (Fig. 3C).

Furthermore, the collagen degradation of CCCS subjected to collagenase was also evaluated based on the methods described in *Materials and Methods*, and was decreased with the increase of either collagen or chitosan concentrations in CCCS (Fig. 3D). Similarly, analysis of the rhaFGF releasing rate showed that both collagen and chitosan concentrations positively inhibited the release rate of rhaFGF from CCCS (Fig. 3E).

Pre-clinical evaluation for CCCS containing rhaFGF in diabetic wound healing

Based on the above observation, we found the best condition of CCCS being 4.1 mg/ml collagen crosslinked with chitosan at 1:1 ratio of it to chitosan to produce the smallest interval porosity and slowest releasing rate of FGF from CCCS, and also the most resistance to collagenase degradation. Therefore, we have made CCCSs containing either low (1 μg/2.5 cm^3) or high (4 μg/2.5 cm^3) rhaFGF as CCCS/FGF(L) or CCCS/FGF (H) to treat diabetic skin wound. Fig. 4A summarizes the healing time of diabetic wound by different treatments. It can be summarized as: (a) general patterns for the healing times are similar to results indicated in Fig. 2D, showing that diabetes (2 months after the onset of hyperglycemia) significantly delayed the wound healing time from 14 days in non-diabetic rats to more than 28 days (Fig. 4A); (b) treatment with either CCCS alone or FGF alone could slightly improve wound healing, showing the healing time being 27 days or to 21~23 days; (c) CCCS/FGF, in particular CCCS/FGF(H), significantly shorten the healing time to ~14 days (Fig. 4A), which is 4–7 days shorter than those collagen sponge with FGF (Fig. 2D). The unhealed wound sizes in different groups at different treating times are summarized in Table 2, which shows similar patterns to healing times in these groups (Fig. 4A). Representative images of wound healing statues from control, diabetes and diabetes treated by CCCS/FGF(H) are shown in Fig. 4B.

Normally, dermal recovery is assessed for three stages: proliferation, remodeling, and maturation. Histopathological examination with HE staining showed that the CCCS/FGF(H)-treated wounds exhibited advancement in all these three stages (Fig. 4C). Several key parameters including squamous metaplasia, inflammation, red blood cells (RBC), new blood capillary, fibroblast, and fibrosis in the different groups are summarized in Table 3, which clearly shows that CCCS/FGF (H) had advanced to the fibroblast-rich stage while the control and diabetic wounds still presented a continuous infiltration of neutrophils and polymorphonuclear cells between days 14 and 28. These examinations revealed that an unstructured type of dermis in the diabetes treated with saline and even with others were unorganized in contrast to those in the CCCS/FGF(H)-treated wounds, showing new dermis formed in an organized and well layered manner.

Since collagen formation is a critical step for the wound healing, we have stained the skin tissue with Masson's trichrome that can highlight the collagen remodeling and maturation. In the groups treated for 7 and 14 days, Masson's trichrome staining showed that CCCS/FGF(H)-treated wounds had more mature collagen development compared to those treated with others (Fig. 5, Data not shown except for those shown in this figure). To support the results of Masson's trichrome staining, quantitative analysis of the critical marker of fibrosis TGF-β1 expression by Western blotting assay showed that CCCS/FGF(H) treatment induced the earliest and highest TGF-β1 expression among groups, and an even higher than that in non-diabetic rats treated with saline at day 7 after starting treatment (Fig. 6). Immunochemical staining of the wound skin for the expression of TGF-β1 supported Western blotting results (Fig. 7A).

To explore the cellular mechanisms for the acceleration of wound healing by CCCS/FGF(H), PCNA expression as a marker of cell proliferation was examined by Western blotting. It was shown that PCNA expression in the wound skin of diabetic rats treated with CCCS/FGF(H) was the highest at day 7 after treatment among all groups (Fig. 6). Immunohistochemical

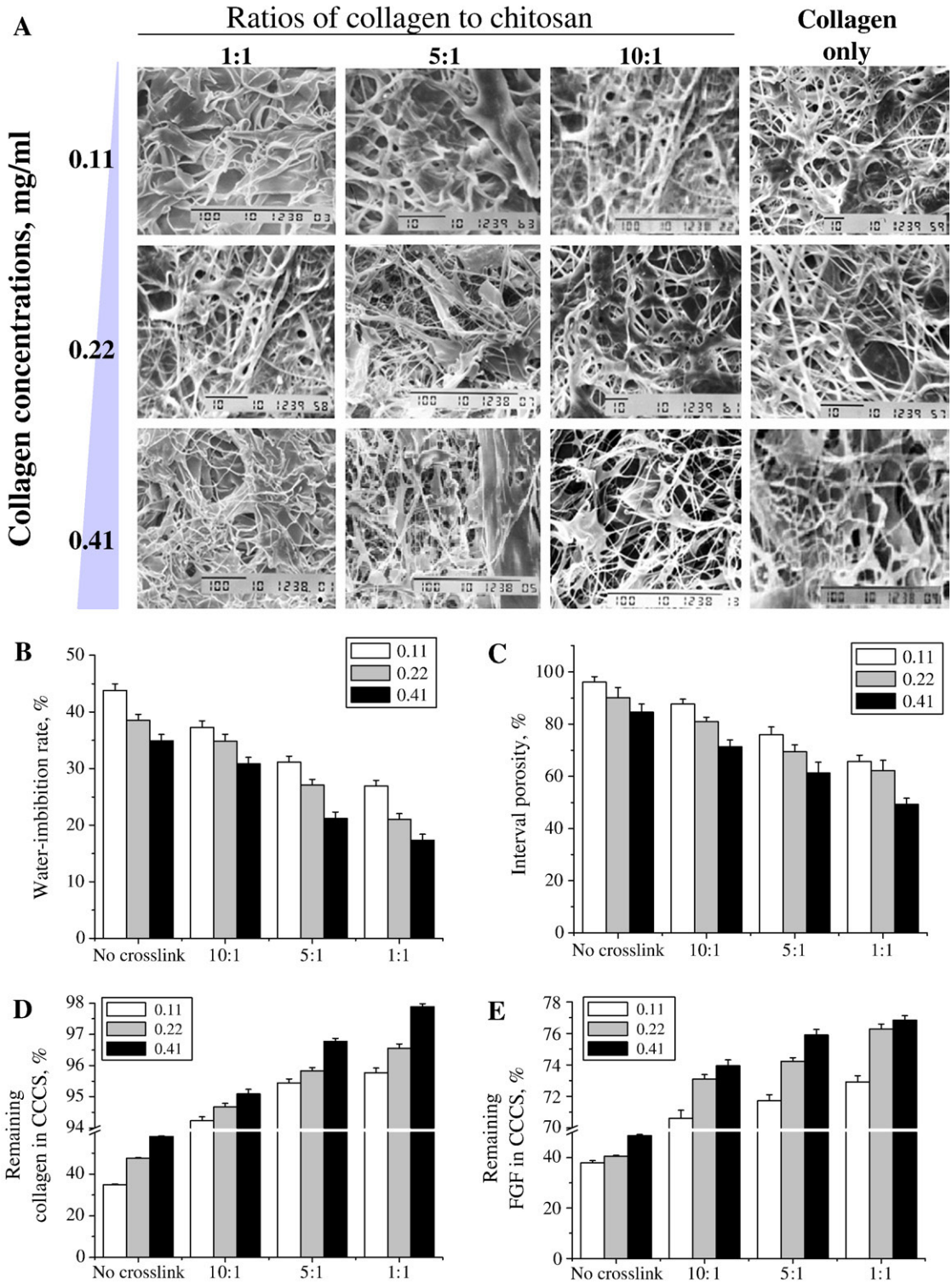

Fig. 3. General evaluation of CCCS for its ultrastructure (A), observed under SEM, water-imbibition (B), interval porosity (C), resistance to collagenase digestion for 60 days (D), and releasing rate of FGF from CCCS/FGF (E).

staining (representative images are given in Fig. 7B) confirmed the results of Western blotting assay, showing that PCNA positive cells are the highest in the wound skin of the diabetic rats treated with CCCS/FGF(H) at day 7 after treatment (control, Fig. 7B).

Discussion

In the present study, we have improved the wound dressing by crosslinking collagen sponge with chitosan. The CCCS made of 4.1 μg /ml collagen crosslinked with equal weight of

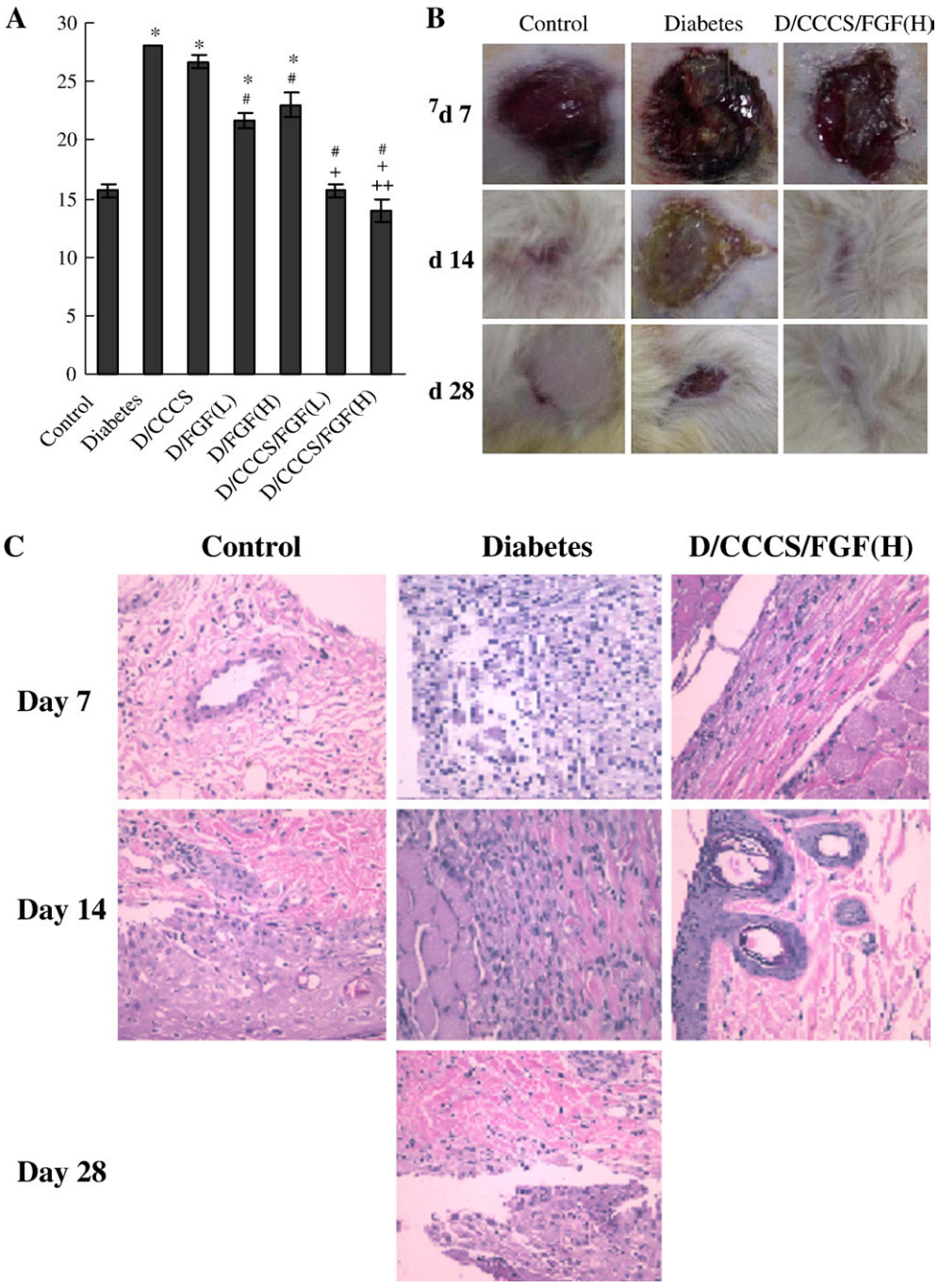

Fig. 4. Pre-clinical evaluation of CCCS/FGF for the therapeutic effects on diabetic wound healing. SD rats were induced to be hyperglycemic and skin wound was made in these diabetic rats at 2 month after hyperglycemia, as described in Fig. 2. Diabetic rats with skin wound were treated under different conditions as indicated in panel A and their definitions are described in the text. Panel A indicates the healing time, and panel B shows the representative images of wound healing status in control, diabetes and diabetes treated with CCCS/FGF(H). Panel C shows the representatively pathological images of the wound skin by H&E staining from control, diabetes and diabetic wound treated with CCCS/FGF(H). *, $p<0.05$ vs control; #, $p<0.05$ vs Diabetes; +, $p<0.05$ vs corresponding D/FGF groups; ++, $p<0.05$ vs D/CCCS/FGF(L).

1% chitosan showed the low imbibition, small interval porosity and high resistance to degradation by collagenase, and slow releasing rate of rhaFGF from CCCS. All these features are required for the qualified wound dressing to accelerate wound-healing process. CCCS/FGF has offered the best healing process for the healing-impaired skin wound in STZ diabetic rats, showing the shortest healing time as 14 days, which is similar to, or even faster than, those in non-diabetic rats treated with saline.

Diabetes-impaired healing process is complex, and includes vascular, neuropathic, immune function, and biochemical abnormalities (Blakytny and Jude, 2006; Brem and Tomic-Canic, 2007). Experimental studies have shown that these pathogenic features of the healing-impaired skin wound

Table 2
Unhealed wound size (mm^2) at different times after starting treatments

Day/group	Control (n=10)	Diabetes (n=10)	D/CCCS (n=10)	D/FGF(L) (n=10)	D/FGF(H) (n=10)	D/CCCS/FGF(L) (n=10)	D/CCCS/FGF(H) (n=10)
0	254	254	254	254	254	254	254
3 d	211.6±8.1*	237.7±16.2	230.2±5.1*	206.7±8.9*	211.3±10.9*	144.4±5.1*#+	139.4±5.8*#+
7 d	122.9±6.3*	219.9±11.9	179.6±7. 2*	159.1±5.6*	155.5±6.7*	87.9±4.3*#+	77.6±3.8*#+
10 d	82.1±4.1*	203.3±11.9	147.9±6.8*	124.3±3.9*	135.7±5.1*	69.1±3.5*#+	47.9±2.5*#+
14 d	23.4±4.3*	180.1±8.8	111.5±5.3*	80.5±3.1*	98.5±4.2*	30.1±2.9*#+	15.8±1.9*#+
18 d	0	163.3±6.7	88.6±4.5**	66.7±2.9*	77.5±4.1*	0	0
21 d		133.5±8.5	55.6±3.9*	44.2±2.3*	51.2±2.9*		
25 d		99.1±24.1	30.5±3.8*	26.8±1.9*	23.5±2.1*		
28 d		66.9±6.1	0	0	0		

Notes: * $p<0.05$ vs Diabetes; # $p<0.05$ vs D/CCCS; + $p<0.05$ vs corresponding D/FGF [i.e.; either D/FGF(L) or D/FGF(H)] same dose comparison).

observed in diabetic patients could be mostly duplicated in the animal models, including genetic and chemical-induced diabetic rats or mice (Tsuboi et al., 1992; Blakytny and Jude, 2006). STZ selectively destroys pancreatic β-cells, inhibits the syntheses and release of insulin, and causes the onset of type 1 diabetes at 2 or 3 days after a single injection of high-dose STZ. The STZ-induced diabetes is typically accompanied by various complications (Seifter et al., 1981; Tsuboi et al., 1992; Schaffer et al., 1997; Cai et al., 2005, 2006; Connelly et al., 2007; Hsueh et al., 2007). Impaired wound healing occurs in patients with diabetes, which was associated with high blood glucose levels (Blakytny and Jude, 2006; Brem and Tomic-Canic, 2007). In the present study, therefore, STZ-induced diabetic rats were used as the model of diabetes to study diabetic wound healing.

Topical application of various growth factors or cytokines directly to stimulate diabetic wound healing has been explored without significant improvement since the quick diffusion and dry of the applied cytokines in the open wound. Several studies have tried to apply collagen sponge including various cytokines to improve the recovery of diabetic wound healing, however they remained not to reach at an optimal goal (Marks et al., 1991; Ono et al., 1999; Nakanishi et al., 2005; Nagato et al., 2006). To consistent with these studies, we found in the present study that collagen sponge showed an unsatified FGF-releasing results since collagen sponge containing different concentrations of FGF did not show the dose-dependent therapeutic effects and only provided a mild improvement (Fig. 2D). We assumed that the sponge can not hold the cytokines very well, and the quick releasing cytokines from collagen sponges may be predominantly responsible for the mild improvement without dose-dependent therapeutic effects.

Application of the chitosan hydrogel on open wounds was recently found to induce significant wound contraction, thereby accelerating wound closure and the healing process (Obara et al., 2005b). In addition, the chitosan hydrogel showed controlled release ability for various growth factors, serving as a novel carrier and inducing neovascularization in vivo. When an chitosan containing bFGF was applied for the wound healing in *db/db* mice, a significant effect was shown on granulation tissue formation, infiltrating cells, and capillary number, but only a minor effect was shown on the degree of reepithelialization (Obara et al., 2003, 2005a).

In the present study, therefore, we have expored the collagen sponge crosslinked with chitosan containing different concentrations of FGF to examine whether this combination could

Table 3
General pathological examinations in the tumor tissues by H&E staining

	SM	Inflammation	New blood capillary	RBC	Fibroblast	Fibrosis
Control (7 day)	−−	++	+	+	+	++
Control (14 day)	+	−	−	−	−	−
Diabetes (7 day)	−	++	+	+	++	−
Diabetes (14 day)	−	++	+	+	+	+
Diabetes (28 day)	±	−	−	+	−	++
D/collagen (7 day)	−	++	++	++	++	−
D/collagen (14 day)	−	+	+	+	−	++
D/collagen (28 day)	+	−	−	−	−	−
D/FGF(L) (7 day)	−	++	++	++	+	+
D/FGF(L) (14 day)	−	+	+	+	−	++
D/FGF(L) (28 day)	+	−	−	−	−	−
D/FGF(H) (7 day)	−	++	++	++	+	+
D/FGF(H) (14 day)	−	+	+	+	−	++
D/FGF(H) (28 day)	+	−	−	−	−	−
D/CCCS/FGF(L) (7 day)	−	++	+	+	+	+
D/CCCS/FGF(L) (14 day)	+	−	−	−	−	−
D/CCCS/FGF(H) (7 day)	−	++	+	+	+	+
D/CCCS/FGF(H) (14 day)	+	−	−	−	−	−

Notes: SM, squamous metaplasia; RBC, red blood cells; + and ++ indicate evident and remarkable evident.

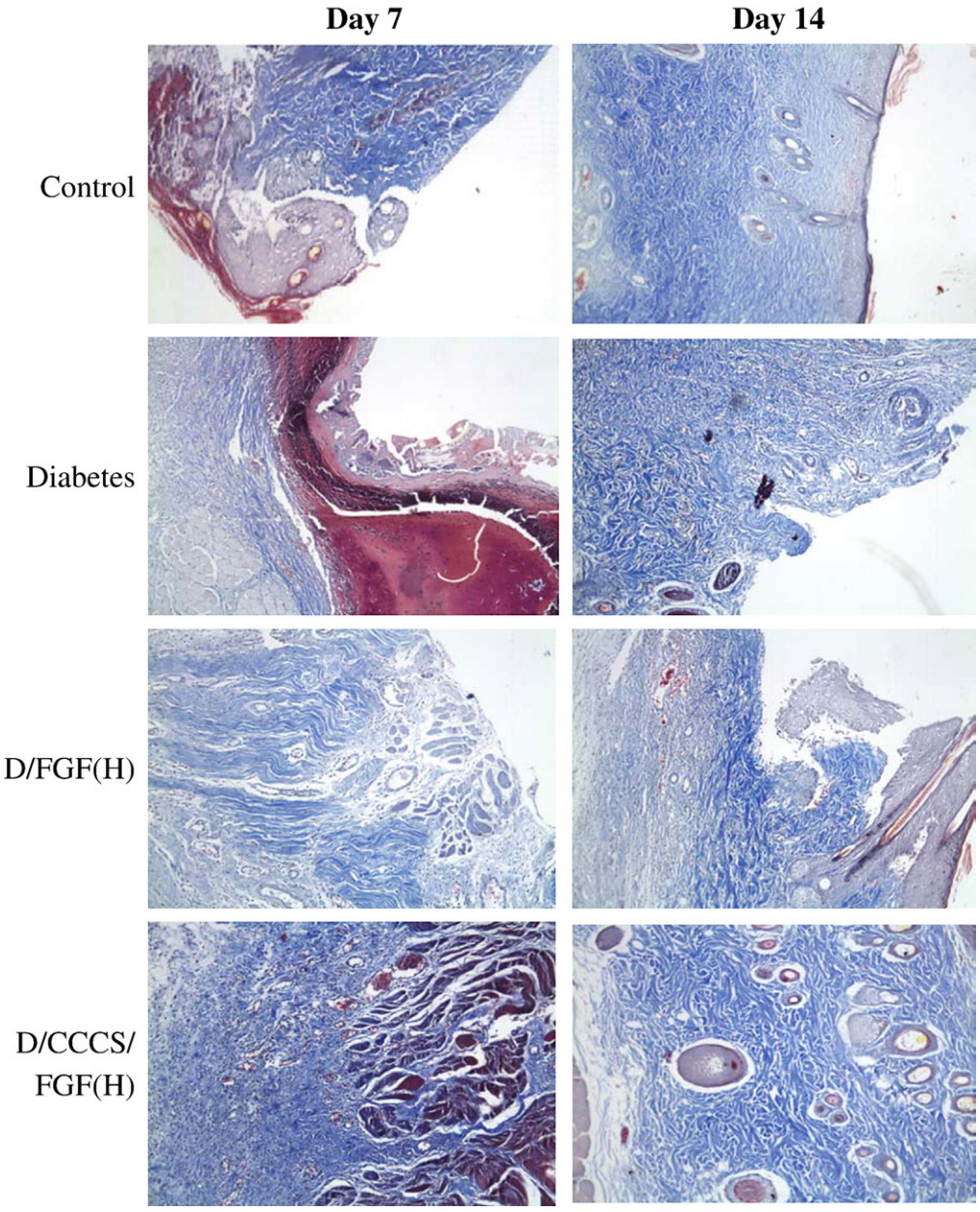

Fig. 5. Masson's trichrome staining for collagen. Tissue sections from wound skin of various groups were stained with Masson's trichrome staining for collagen formation. The representative images from groups of control, diabetes, D/FGF(H) and D/CCCS/FGF(H) at day 7 and day 14 after treatment are given. The rest images at different time of these four groups and others of other groups are not shown.

solve the disadavantage of collagen sponge alone (Marks et al., 1991; Inoue et al., 1998; Ono et al., 1999; Lee, 2005; Nakanishi et al., 2005; Nagato et al., 2006) or chitosan alone (Ishihara et al., 2001; Degim et al., 2002; Azad et al., 2004; Alemdaroglu et al., 2006). Both collagen and chitin (chitosan is one of soluble derivatives of chitin) are amongst the most abundant polymers in life, and do not exist together as blends in nature. However, both have intrinsic properties that provide a strong, but manipulable scaffolding structure, which are required for producing artificial blends that confer unique structural and mechanical properties. One of the most promising features of chitosan is its excellent ability to be processed into porous structures. Therefore, the preparation and characterization of a collagen crosslinked with chitosan have been previously explored (Taravel and Domard, 1993, 1995, 1996). They found that under classical conditions, there existed a competition between collagen gelatin and the formation of a pure polyanion-polycation complex with chitosan (Taravel and Domard, 1993). They have systemically characterized the physical and biochemical features for the interaction of collagen and chitosan. More recently, Sionkowska et al. (2004) have further investigated the molecular interactions in collagen and chitosan blends. They found that collagen and chitosan are miscible at the molecular level and exhibit interactions between the components, and the collagen helix structure is lost in collagen/chitosan films with increasing chitosan content. The

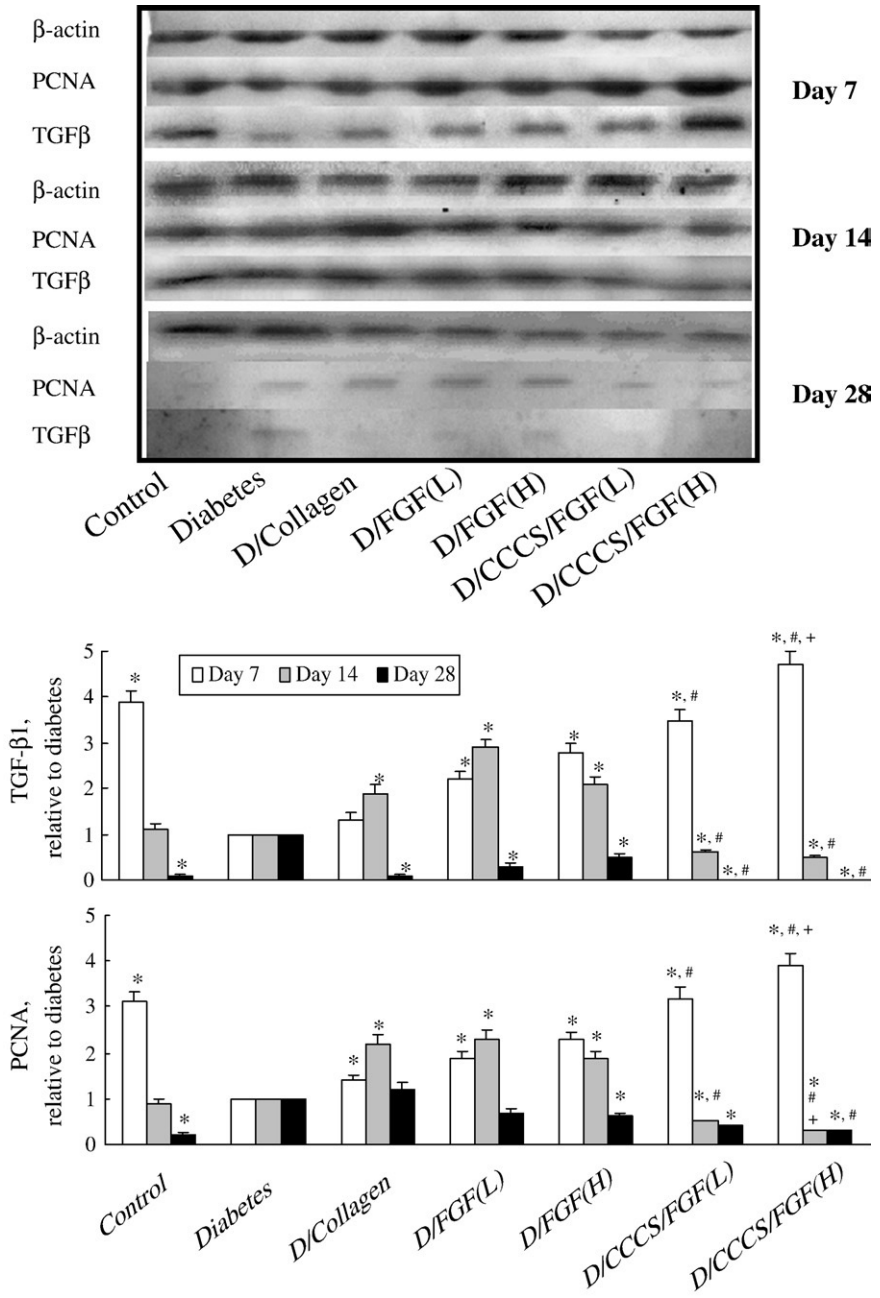

Fig. 6. Western blotting assay for TGF-β1 and PCNA expressions. Total proteins were prepared from the wound skin of various groups of diabetic rats with different treatments at different post-treatment times (7 to 28 days), and subjected to Western blotting assay for TGF-β1 expression and PCNA expression. The band density of target proteins was quantitatively analyzed and presented as relative to diabetic group. β-actin expression was used as protein loading control. *, $p<0.05$ vs diabetes; #, $p<0.05$ vs corresponding D/FGF groups; +, $p<0.05$ vs D/CCCS/FGF(L).

blending of collagen with chitosan gives the possibility of producing new materials for potential biomedical applications. Tangsadthakun et al. (2007) also indicated that low-molecular-weight chitosan was more effective to promote and accelerate cell proliferation with more potential to be applied as new materials for skin-tissue engineering. In confirmation and extension of these previous studies, we further demonstrated the improvement of certain biochemical and biological advantages, including less imbibition, small interval pores, resistance to collagenase-cause degradation and low releasing rate of cytokine incorporated into the CCCS.

Several recent investigators have began to show the potential application of collagen/chitosan as matrices for liver tissue engineering (Wang et al., 2003, 2005), cartilage tissue engineering (Shi et al., 2005), periodontal tissue engineering (Zhang et al., 2006), skin-tissue engineering (Tangsadthakun et al., 2007), and even used for immobilization and target delivery of stem cells (Selezneva et al., 2006). To consistent with these recent studies, we provide the first evidence to show the successful use of CCCS/FGF for therapy of the healing-impaired diabetic skin wound. We showed a signficant dose-dependent manner for the imrovement of diabetic wound

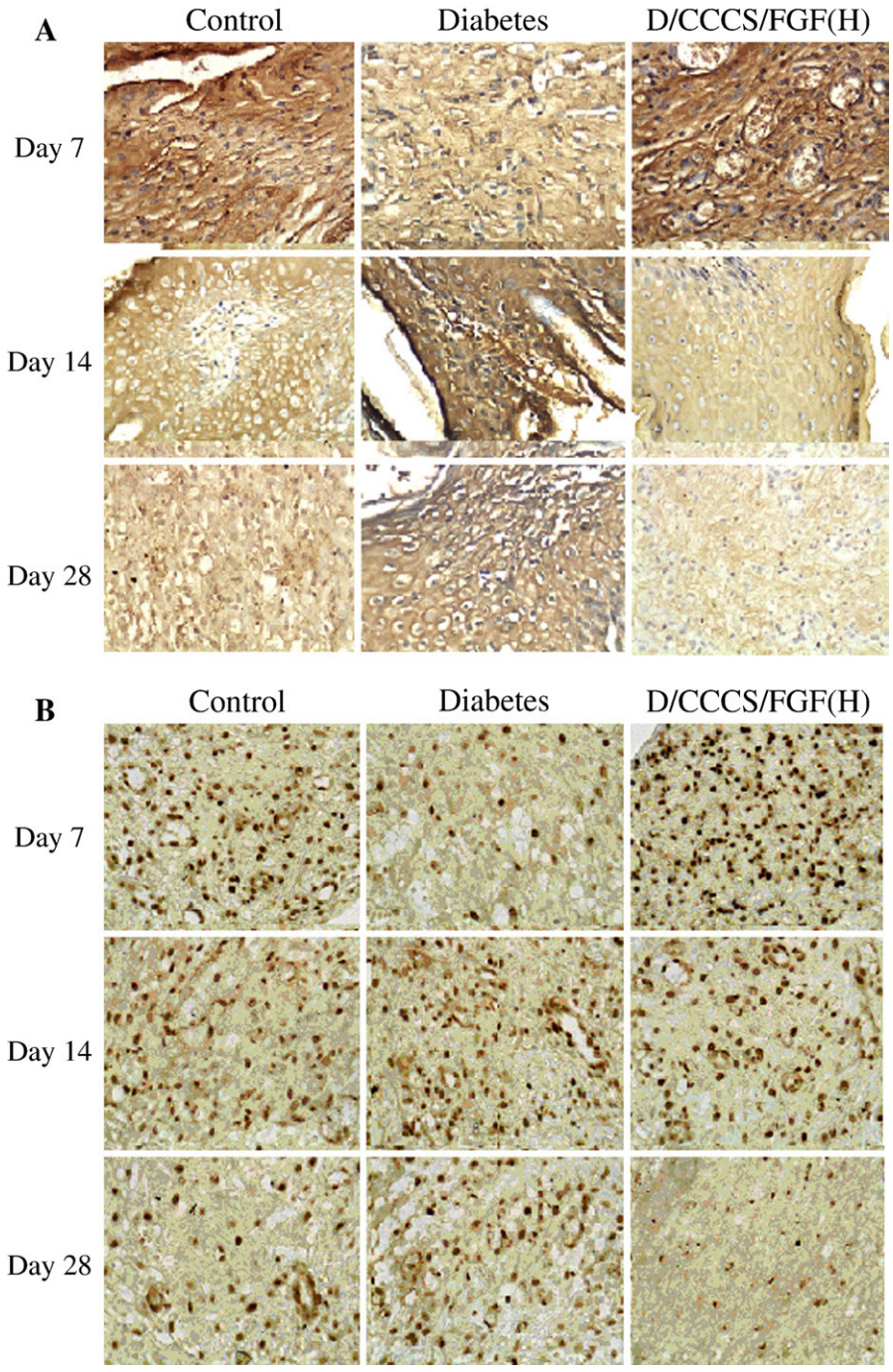

Fig. 7. Immunohistochemical staining for TGF-β1 and PCNA expressions. Tissues were prepared from different groups at different post-treatment times as described in *Materials and Methods*, and immunohistochemically stained for TGF-β1 (A) and PCNA (B) with their specific antibodies. The images shown in this figure are the representatives of three groups of control, diabetes and diabetes treated with CCCS/FGF(H) at indicated post-treatment times. The rest images from these groups and other groups were not shown.

healing, and the improved therapy of CCCS/FGF(H) was also signficantly better (healing time as 14 days) than collagen sponge alone containing FGF at high concentration (healing time as 21 days). The signfciant improvement for the diabetes-impaired wound healing was not only evident in the wound healing time and size, but also supportive in the molecular levels, such as TGF-β1 up-regulation and cell proliferation, both of which are critical steps for the recovery of wound skin. Therefore, this finding opens an avenue for us to clinically explore the improvement of diaebtic wound healing with the new wound dressing to contain an optimal combination of cytokines and growth factors.

Conclusion

In summary, collagen crosslinked with chitosan provided several advantages required for wound dressing. Pre-clinical studies using type 1 diabetic rats with trauma skin wound

indeed showed the most efficiently therapeutic effect of the new wound-dressing containing FGF (i.e.: CCCS/FGF), i.e.: the shortest healing time (14 days in the CCCS/FGF-treated group as compared to 18～21 days in other treatment groups), the quickest tissue collagen generation, and the earliest and highest TGF-β1 expression and dermal cell proliferation (PCNA expression), as compared to other treatments. All these results suggest that CCCS/FGF may be an ideal wound dressing to accelerate the wound healing potentially for diabetic patients.

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